

FORM PTO-1390  
(REV. 10-2000)U.S. DEPARTMENT OF COMMERCE  
PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

20276P

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

**09/762794**INTERNATIONAL APPLICATION NO.  
PCT/US99/17931INTERNATIONAL FILING DATE  
10 AUGUST 1999PRIORITY DATE CLAIMED  
14 AUGUST 1998

TITLE OF INVENTION

PROCESS DELIVERY SYSTEM

APPLICANT(S) FOR DO/EO/US

ROBERT S. LOWE, KATHRIN U. JANSEN, JOSEPH J. JOYCE, WILLIAM L. MC CLEMENTS, JAMES C. COOK  
JESSICA CHING-YEE LING, MICHAEL P. NEEPER**Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:**

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures [35 U.S.C. 371(f)] at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(i).
4. ☒ A proper Demand for International Preliminary Examination was made and the US was elected by the expiration of the 19th month from the earliest claimed priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed [35 U.S.C. 371(c)(2)].
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ has been communicated by the International Bureau.
  - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed [35 U.S.C. 371(c)(2)].
7. ☒ Amendments to the claims of the International Application under PCT Article 19 [35 U.S.C. 371(c)(3)].
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)].
9. ☐ An oath or declaration of the inventor(s) [35 U.S.C. 371(c)(4)].
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 [35 U.S.C. 371(c)(5)].

**Items 11 to 16 below concern other document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ Other items or information:



**EXPRESS MAIL CERTIFICATE**  
 DATE OF DEPOSIT FEB 9 2001  
 EXPRESS MAIL NO. EL523906936 US  
 I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS  
 BEING DEPOSITED WITH THE UNITED STATES POSTAL  
 SERVICE AS EXPRESS MAIL "POST OFFICE TO  
 ADDRESSEE" BEFORE 5 P.M. ON THE ABOVE DATE IN  
 AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER  
 FOR PATENTS, WASHINGTON, D.C. 20591  
 MAILED BY 15-000000  
 DATE 12-9-01

U.S. APPLICATION NO. (if known, see 37 CFR 1.53)

INTERNATIONAL APPLICATION NO.

ATTORNEY'S DOCKET NUMBER

09/762794

PCT/US99/17931

20276P

17. ☒ The following fees are submitted:**BASIC NATIONAL FEE [37 CFR 1.492(a)(1)-(5)]:**

Neither international preliminary examination fee (37 CFR 1.482)

nor international search fee [37 CFR 1.445(a)(2)] paid to USPTO

and International Search Report not prepared by the EPO or JPO.....\$1,000.00

International preliminary examination fee (37 CFR 1.482) not paid to

USPTO but International Search Report prepared by the EPO or JPO.....\$860.00

International preliminary examination fee (37 CFR 1.482) not paid to

USPTO but international search fee [37 CFR 1.445(a)(2)] paid to USPTO.....\$710.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)

but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$690.00

International preliminary examination fee paid to USPTO (37 CFR

1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$100.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30  
months from the earliest claimed priority date [37 CFR 1.492(e)].

\$130.00

Claims	Number Filed	Number Extra	Rate		
Total Claims	12 - 20 =	0	X \$18.00	\$0.00	
Independent Claims	3 - 3 =	0	X \$80.00	\$0.00	
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$0.00	

**TOTAL OF ABOVE CALCULATIONS =**

\$230.00

☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated  
above are reduced by 1/2.**SUBTOTAL =**

\$230.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20☐ 30 months from the earliest claimed priority date [37 CFR 1.492(f)].

+

\$0.00

**TOTAL NATIONAL FEE =**

\$230.00

Fee for recording the enclosed assignment [37 CFR 1.21(h)]. The assignment must be  
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property. +**TOTAL FEES ENCLOSED =**

\$230.00

Amount to be  
refunded  
charged

- a. ☐ A check in the amount of \$ \_\_\_\_\_ to cover the above fees is enclosed.
- b. ☒ Please charge my Deposit Account No. 13-2755 in the amount of \$230.00 to cover the above fees.  
A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any  
overpayment to the Deposit Account No. 13-2755. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive  
[37 CFR 1.137(a) or (b)] must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

MERCK & CO., INC.  
Patent Department, RY60-30  
P.O. Box 2000  
126 East Lincoln Avenue  
Rahway, New Jersey 07065-0970

DATE: February 9, 2001

PHONE #: (732) 594-3046

*Joanne M. Giesser*  
SIGNATURE

JOANNE M. GIESSER  
NAME

32,838

REGISTRATION NUMBER

PROTEIN DELIVERY SYSTEM USING HUMAN PAPILLOMAVIRUS  
VIRUS-LIKE PARTICLES

FIELD OF THE INVENTION

5 This invention is related to a method of delivering protein to a cell by fusing it to a modified papillomavirus L2 protein, and expressing the fused protein in a virus-like particle. The invention is also directed to modified genes encoding the proteins, the modified virus-like particles and host cells containing the virus-like particles.

BACKGROUND OF THE INVENTION

Human Papillomaviruses (HPVs) infect the genital tract and have been associated with various cancers and other diseases. Recombinantly produced L1 protein and L1 + L2 protein can self-assemble to form virus-like particles (VLPs). These VLPs are immunogenic, and may be the basis of a vaccine.

15 Currently, however there is no known prophylactic vaccine or ideal therapeutic treatment of disease caused by HPV. An immune response to other HPV proteins co-delivered within the context of VLPs such as HPV early proteins or genes may enhance the protective effect of a vaccine. Recently, a paper by Greenstone et al described VLPs in which the L2 protein was modified to create a fusion protein between full length L2 and HPV E2 or E7. (Greenstone et al, 1998 *Proc. Natl. Acad. Sci. USA* 95:1800-1805.) The fusion proteins co-assembled with L1 into VLPs which appeared indistinguishable morphologically from L1 VLPs and in their ability to elicit neutralizing antibodies. Furthermore, in tumor challenge models, mice vaccinated with chimeric L1 + L2:E7 fusion VLPs were found to protect mice from E7-expressing tumors.

20 However, there are potential problems associated with the use of a full-length L2 as a fusion partner. First, due to size restraints, an entire L2 as a fusion partner has a limited capacity to accept large fusion proteins. For instance, fusion of E1 with intact L2 of CRPV leads to morphologically aberrant VLPs. Furthermore, expression of a full-length L2 will most likely affect the activity of enzymatically active proteins and therefore may limit its usefulness to deliver functional proteins into a cell. The minimal sequence requirements of L2 enabling coassembly with L1

into VLPs are unknown and the ability to delete significant portions of L2 while retaining the capacity to assemble with L1 is also unknown.

- It would be desirable to provide a minimal L2 fusion protein which still retains the protein delivery function of a full-length L2, yet can deliver larger and functionally-active proteins.

## DESCRIPTION OF THE INVENTION

- This invention is directed to a method of delivering a peptide or protein to a cell comprising the steps of: a) fusing a nucleic acid sequence encoding the peptide or protein to be delivered to a modified papillomavirus (PV) L2 gene to create a fusion protein gene, wherein the L2 gene is less than full-length and comprises at least the coding sequences for the amino-terminal 69 amino acids and the carboxy-terminal 84 amino acids (aa) of L2; b) expressing the fusion protein gene in a host cell to obtain a fusion protein; c) contacting the fusion protein with PV L1 protein under conditions wherein the fusion protein and the L1 protein spontaneously combine to form a virus-like particle (VLP); and d) delivering the VLP to a cell.

- This invention also relates to nucleic acids encoding a fusion protein comprising a less than full-length L2 protein fused to a gene encoding a peptide or protein of interest, and to these fusion proteins. In particular, this invention is related to L2 fusion proteins which comprise less than a full-length L2, and comprise at least the amino terminal 69 amino acids and the carboxy terminal 84 amino acids of L2. Additionally, this invention relates to the virus-like particles (VLPs) which contain the fusion protein, and to cellular hosts containing these VLPs.

## DESCRIPTION OF THE DRAWINGS

- Figure 1 shows the assembly of the minimal L2 DNA construct from native HPV16 L2. The coding sequence of HPV16 L2, shown in lower case italics, served as template for PCR amplifications. The initiator and terminator codons are in uppercase. Sense primers, I and A, are shown above the template; anti-sense primers, C and D, below. New sequence information,

introduced by PCR amplification are shown in uppercase. The complimentary sequences in primers A and C are underscored.

Figure 2 is the amino acid translation of the HPV 16 Minimal L2 Gene. Amino acid changes resulting from the introduction of the polylinker containing unique restriction sites Not I, Sac I, and Xho I are indicated by italics. Amino acids 1-69 are contributed from the amino terminus of L2. A glutamic acid residue is added at position 70. Amino acids 71-154 are contributed from the carboxyl terminus of L2. At position 72, a glutamic acid residue replaces a serine found in the full-length wildtype L2.

Figure 3 is a Western blot of immunoprecipitated yeast lysates, showing co-immunoprecipitation of L2-E chimeric proteins with HPV16 L1 VLP-specific Mab H16:V5. The left panel shows anti-E1 stained immunoblot of minimal L2:E1 fusion (mL2:E1) clones E1-121 to E1-124, mL2:E2 sample E2-23 and no lysate samples are specificity controls. The middle panel shows anti-E7 stained immunoblot of minimal L2:E7 fusion (mL2:E7) clones E7-125 to E7-127 and E7-31; mL2:E2 sample E2-23 and no lysate samples are specificity controls. The right panel shows anti-E2 stained immunoblot of mL2:E2 fusion clones E2-33a, E2-33b; sample E2-33b is also shown with no H16:V5 capture antibody as a control and YP3#1 no E protein control is shown as mL2 vector.

Figure 4 is a table listing beta galactosidase activities of transiently transfected cell lysates.

#### DETAILED DESCRIPTION OF THE INVENTION

The method of this invention can be used with virtually any papillomavirus strain. In preferred embodiments, the HPV is one of the strains which is associated with genital warts and/or genital cancers, and in particular may be selected from the group consisting of: HPV6a, HPV6b, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, and HPV56.

Wild-type virus-like particles are predominantly made of L1 protein, although they contain a minor amount of L2 protein. In

accordance with this invention, the VLPs have been modified so that they still contain wild-type L1 protein, but now contain a deleted L2-fusion protein instead of wild-type L2. The L2 segment of the fusion protein is substantially less than the full length L2 protein; it contains peptide

domains from the amino terminus and from the carboxyl terminus which are necessary for association with L1 proteins and incorporation into VLPs, at the least, for HPV type 16, the coding sequences for the amino-terminal 69 amino acids and the carboxy-terminal 84 amino acids (aa) of L2. The L2 sequence may be larger than this, as long as it is less than wild-type. Preferably the total amount of L2 amino acids present is less than about 60% of wild type, more preferably less than about 50% of wild-type, and even more preferably less than about 35% of wild type. This modified L2 will be referred to throughout the specification and claims as a "minimal L2" gene and protein.

Virtually any protein or peptide can be fused to the minimal L2 protein to make the fusion protein. HPV proteins which are normally not part of the VLP are preferred if the goal is to make a VLP which induces an increased immune response as compared to a wild-type VLP. For this application, these proteins include but are not restricted to HPV: E1, E2, E3, E4, E5, E6, and/or E7, HIV TAT and/or beta lactamase. Particularly preferred proteins are E1, E2, and E7. These fusion proteins and the nucleic acids encoding them make up another aspect of this invention.

One of the advantages of this invention is that the fusion partner may be a large protein. For example, the fusion partner may be over 50 kD, over 60 kD and even over 70 kD. This increased size allows for the introduction of most known proteins, and makes this delivery system particularly useful not only for HPV-related proteins as described above, but also for proteins which are not associated treatment of with HPV disease.

Thus, this invention includes methods of using a modified VLP as a generalized transporter, directing the delivery of virtually any desired protein or peptide into cells. In one embodiment, reporter or regulatory proteins are delivered to cells as a measure of VLP uptake by cells. As an example of fusion candidates which is by no means limiting, is the regulatory protein of HIV known as TAT. Fusions of TAT to the minimal L2 have been shown to retain protein function as well as form VLPs with L1. This is useful in establishing neutralization assays.

The fusion protein and L1 protein may be made simultaneously by transforming a selected host cell with genes encoding the proteins under conditions which allow expression of the two genes. General techniques, the genes for L1s and L2, and methods for recombinant expression of L1 and L2 proteins are known in the art, and may be used. Preferred host cells include yeast, insect cells, mammalian cells and *E. coli*.

Under one preferred embodiment of this invention, yeast are transformed with plasmid DNA containing the genes encoding L1 and the L2 fusion under control of known yeast promoters, such as the yeast Gal 1 and Gal 10 promoters. Expression of each gene product is induced by addition of galactose to the growth media, and the VLPs are isolated from the induced cell lysates.

Alternatively, the one set of host cells may be transformed to express only L1 protein, and a second set of host cells may be transformed to express the L2 fusion protein of this invention. The respective proteins are harvested, optionally subjected to a purification step, and are brought into contact so that VLPs containing the fusion protein are formed.

The modified VLPs make up yet another aspect of this invention. These VLPs may be administered as a vaccine, or as part of a therapeutic regimen. Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose PV infections. The effective amount may vary according to a

variety of factors such as the individual's condition, weight, gender and age. Other factors include the mode of administration. Generally, the compositions will be administered in dosages ranging from about 1 mcg to about 1 mg.

5           The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneously, topically, orally, mucosally, intravenously and intramuscularly.

          The vaccines of the invention comprise DNA, RNA or proteins encoded by the DNA that contain the amino-terminal and  
10   carboxyl-terminal portions of L2 which permit the incorporation of proteins fused to it to be incorporated into VLPs. Such vaccines are also safe enough to be administered without danger of clinical infection, do not have toxic side effects, can be administered by an effective route, are stable, and are compatible with vaccine carriers.

15           The vaccines may be administered by a variety of routes, such as orally, parenterally, subcutaneously, mucosally, intravenously or intramuscularly. The dosage administered may vary with the condition, sex, weight, and age of the individual; the route of administration; and the type of PV of the vaccine. The vaccine may be used in dosage forms such  
20   as capsules, suspensions, elixirs, or liquid solutions. The vaccine may be formulated with an immunologically acceptable carriers or adjuvants, or other excipients.

          The vaccines are administered in therapeutically effective amounts, that is, in amounts sufficient to generate an immunologically  
25   protective response. The therapeutically effective amount may vary according to the type of PV. The vaccine may be administered in single or multiple doses.

          The purified proteins of the present invention may be used in the formulation of immunogenic compositions. Such compositions, when  
30   introduced into a suitable host, are capable of inducing an immune response in the host.



The following non-limiting Examples are presented to better illustrate the invention.

### EXAMPLE 1

#### Construction of the Modified L2 Gene

The modified HPV16 L2 gene was constructed from the coding sequences for the amino-terminal 69 amino acids and the carboxy-terminal 84 amino acids (aa) of HPV16 L2 which were fused in frame by a synthetic polylinker that introduced unique Not I, Sac I, and Xho I restriction endonuclease recognition sites and resulted in the insertion of one glutamic acid residue and the mutation of a serine residue to glutamic acid. (Figure 1).

PCR primers (Midland Certified Reagents; Midland, TX) were designed to amplify L2 sequences from the native L2 gene contained within the vector, pGal110 (Hofmann, K, et al 1995 *Virology* 209:506-518) into which the genes encoding HPV16 L1 +L2 were inserted. (See Figure 1).

Primers I ( 5'- CTT CCC CCC GGG CAC AAA ACA AAA TGC-3'; SEQ.ID.NO. 1) and C (5'- CTC GAG CTC GCG GCC GCC TGT ACC CGA CCC-3'; SEQ.ID. NO. 2) amplified a 265 base pair (bp) sequence encoding the amino-terminal 69 aa and 23 bp of upstream untranslated sequence including a Sma I restriction enzyme site. Primer C modified and extended the L2 amino terminal-encoding region and appended Not I, Sac I and Xho I restriction enzyme sites downstream of the L2-encoding sequences.

Primers A (5'- GCG GCC GCG AGC TCG AGG GTT ATA TTC CTG CAA ATA CAA-3'; SEQ.ID.NO. 3), C and D (5'-CCC TCC AGA TCT CTA GGC AGC CAA AGA GAC ATC TG-3' SEQ.ID.NO. 4) amplified a 285 bp sequence encoding the carboxy-terminal 84 aa of L2 plus 6 bp which added a Bgl II restriction enzyme site. Primer A also appended a

17 bp sequence containing Not I, Sac I, and Xho I sites upstream of the L2-encoding sequence.

The minimal L2 expression construction was assembled through complementary sequences added by primers A and C. The isolated DNA products of the I/C and A/D amplification reactions above were both used in a PCR reaction which included the I and D primers. To facilitate the joining of the fragments through their 17 bp complementary sequence, three PCR cycles were performed with the annealing temperature at 37°C, followed by 15 cycles with the annealing temperature of 57°C. The resulting amplification product was blunt-end ligated into pcrScript (Stratagene, LaJolla, CA) and transformed into XL-1 Blue MRF<sup>+</sup> cells (Stratagene). Positive clones were identified by PCR using primers I and D, and confirmed by restriction digest analysis. The construction was then verified by automated sequence analysis (Perkin Elmer, Inc., Foster City, CA).

Plasmid DNA from an appropriate isolate was then digested with Sma I and Bgl II; a fragment of approximately 0.5 kilobase pairs (kb) was gel purified and ligated with the 14 kb Sma I and Bgl II vector fragment of pGAL110 into which the HPV16L1 gene was inserted adjacent to the GAL 1 promoter. Competent DH5 *E. coli* cells (Gibco BRL, Rockville, MD) were transformed with the ligation mixture and transformants selected on LB ampicillin plates (Remel, Lenexa, KS). Clones were initially screened by PCR in which primers D and I were used to amplify portions of L2. Sequencing of candidate clone YP3#1 verified the sequence to be that as shown in Figure 2.

YP3#1 was then employed as the backbone construct into which genes encoding HPV16 E1, E2, E7 or Tat open reading frames were inserted.

## EXAMPLE 2

### Insertion of HPV E Protein- and Tat-encoding Genes

The gene encoding HPV16 E2 was obtained by PCR amplification of a HPV16 positive clinical sample which was then inserted directly into the subcloning vector pCRII (Stratagene) and sequence verified as above. The E2 gene sequence was then modified in the following manner:

1) In-frame Xho I, Nae I, Not I -containing DNA sequences were added to the amino terminal portion of E2. Additionally, Not I, Nae I, and Xho I-containing sequences were added to the carboxyl-terminal portion of E2 to facilitate insertion within E2 at the Not I, Xho I sites.

2) The DNA sequences were altered by PCR mutagenesis to encode alanine residues encode at residues glutamic acid 39 and isoleucine 73. This was designed to inactivate E2 protein function.

The modified HPV16 E2 gene described above was digested with Not I, Xho I and ligated with similarly digested YP3#1 vector.

Transformants containing the properly-inserted E2 sequences were selected by PCR sequence verified and designated YP3-E2.

A similar approach was employed for inserting the genes encoding HPV16 E1, HPV16 E7 and HIV Tat (creating YP3-E1, YP3-E7 and YP3-Tat, respectively). The construct in which Tat was inserted at the carboxy terminus of minimal L2 was designated as YP3-cTat. For E1, glycine 482 was altered to aspartic acid; for E7, cysteine 24 and glutamic acid 26 were both changed to glycine to inactivate protein function. The resultant constructions were then used to transform yeast.

### EXAMPLE 3

#### Identification and growth of yeast expressing chimeric VLPs

Plasmid DNA of YP3#1 and derivatives described above were used to transform *Saccharomyces cerevisiae* (*MAT $\alpha$* , *leu2-04*, *prb1::HIS3*, *mn9::URA3*, *cir<sup>0</sup>*) by the spheroplast method (Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75:1929-1933). Transformed spheroplasts were plated onto selective (leucine minus) medium (Remel, Lenexa, KS).

Clones were isolated through two rounds of single colony selection. Small liquid cultures of candidate clones were grown to high cell density in medium containing galactose. Crude extracts were prepared by vigorous agitation with glass beads followed by centrifugation. The clarified  
5 extracts were analyzed for expression of L1, the L2 component, and VLPs by various methods including SDS PAGE, ELISA, immunoblotting, and EIA, using monoclonal antibodies or monospecific polyclonal antisera that recognize L1, or L2, or the amino or carboxy termini of L2, or L1 VLPs, or E1, or E2, or E7, or any other protein or peptide fused to the modified L2.  
10 Clones which expressed the L2 component and formed VLPs were selected for further characterization. One-liter or 16-liter cultures of selected clones were grown in galactose containing medium for large-scale preparation of chimeric VLPs.

#### EXAMPLE 4

##### Purification of HPV Type 16 L1/L2<sub>mini</sub>/E2 Chimeric VLPs

20 Cell pellets from a 16-liter fermentation of *S. cerevisiae* were stored frozen at -70°C. Frozen cells "Breaking Buffer" (200 mM MOPS, pH 7, 1 mM CaCl<sub>2</sub>) was added to give approximately 20% (w/v) slurry. BENZONASE® (Nycomed Pharma) was added to 750 units/g wet cell weight. The cell slurry was broken at a pressure of approximately 19,000  
25 psi by 5 passes in a M110-Y Microfluidizer (Microfluidics Corp., Newton, MA). The cell lysate was clarified by microfiltration through a 0.65 micron pore size hollow-fiber cartridge and then diafiltered with three volumes of 0.25 M sodium citrate, 0.2 M MOPS, pH 7.0. Permeate was loaded onto a POROS ® 50HS resin (Perseptive Biosystems, Cambridge,  
30 MA) equilibrated in 200 mM MOPS, pH 7, 250 mM sodium citrate. The column was washed with 50 mM MOPS, 0.5 M NaCl, 5 mM sodium phosphate, pH 7 and eluted with a linear gradient from 0.5 to 1.5 M NaCl

in the same buffer. Column fractions were analyzed by immunoblotting and SDS-PAGE with colloidal Coomassie detection.

The 50HS pool was filtered through a 0.22 mm filter and applied to a ceramic hydroxyapatite (HA) Type II (Bio-Rad) column. The column was washed with 50 mM MOPS, pH 7, 1.25 M NaCl, 5 mM sodium phosphate and eluted with a linear gradient from 5 to 200 mM sodium phosphate, pH 7 in 1.25 M NaCl. Fractions were analyzed by Western blot and SDS-PAGE. Fractions showing comparable purity and enrichment of L1 protein were pooled. The pooled fractions were filtered aseptically through a 0.22 mm membrane and submitted for electron microscopy.

#### EXAMPLE 5

##### Detection of VLPs by Electron Microscopy

Transmission electron microscopy was performed by EMBS (Elkridge, MD). Appropriately diluted samples were placed on a 300 mesh carbon-coated copper grid and allowed to air dry. Grids were stained with phosphotungstic acid. All microscopy was performed using a JEOL 1200 EX transmission electron microscope. The micrographs generated had a final magnification of 138,000 x. Electron microscopy confirmed the presence of intact VLP particles with a mean diameter of 32 nm. These particles were indistinguishable morphologically from yeast-expressed L1 or L1+ L2 particles. Confirmation of the presence of L2 fusions within the VLPs was demonstrated by immunoblotting analysis of process fractions (See Example 6).

#### EXAMPLE 6

##### Co-immunoprecipitation of L2 fusion proteins with L1 specific anti-VLP antibodies.

To show that the L2 fusions were an integral component of the VLPs, VLPs were captured by an HPV16 L1 VLP-specific monoclonal antibody (mAb) H16:V5 (Wang, D. et al. 1997 *J. General Virology*, 78: 2209-2215), washed extensively and the retained products detected by immunoblotting. In brief, magnetic beads precoated with sheep anti-mouse IgG (Dynabeads M-280; Dynal, Oslo) were washed with TMOPS buffer (0.05M MOPS, pH 7.0, 0.4M NaCl, 0.1% Tween-80) containing 5% non-fat dry milk. The washed beads were incubated overnight at 4°C with H16:V5 mAb in the same solution. Unbound mAb was removed by washing with TMOPS. Beads were then incubated with control or VLP-containing crude yeast extracts (see Example 3) or partially-purified VLP preparations (see Example 4). Extracts containing 100-400 µg total protein were diluted with 0.2 MOPS, pH 7.0, 2 mM MgCl<sub>2</sub> and incubated with beads for 4 hours at 4°C. Beads were then washed extensively with TMOPS; bound proteins were recovered by heating the beads at 95°C in Laemmli sample buffer, resolved by SDS-PAGE and identified by immunoblotting.

Identification of the L2 fusion protein was performed with antibodies that recognize the L2 portion of the protein and by antibodies that recognize the protein of interest fused to L2, for example E1, E2, or E7. In addition to the inclusion of control yeast extracts, other controls included 1) beads which have not been incubated with H16:V5, and 2) beads which have not been incubated with yeast extract. Figure 3 shows the immunoblot results of VLPs captured by the H16:V5 Mab and immunoblotted as described above and then stained with anti-E1, anti-E7 or anti-E2 antisera. In each case for multiple isolates, the E-protein:L2 fusions were detected amount the captured, purified VLPs using the appropriate antibody, but not in the absence of lysate or using purified chimeric VLPs containing E-proteins different from the detection antisera.

Coprecipitation of the L2 fusion protein with L1 using a monoclonal antibody specific for L1 VLPs was taken as an indication that the L2 fusion protein was associated with the VLP.

### EXAMPLE 7

#### Demonstration of Tat activity in Tat/minimal L2 fusions in mammalian cells.

- 5 To demonstrate that Tat /L2 fusions retained Tat activity, an expression plasmid was constructed in which DNA encoding the HIV Tat gene was inserted downstream of the minimal L2 gene so that it would be linked to the carboxyl terminus of L2 in the translated product. This DNA cassette was digested with Bgl II and inserted within the Bgl II site of the
- 10 mammalian expression vector, V1Jp (Montgomery, D. et al 1993 *DNA & Cell Biol.* 12:777) adjacent to the CMV promoter. The L2/Tat fusion was created by PCR of the minimal L2 (lacking a translational stop signal) using the oligomers 5'-TCC CCC GGG AGA TCT GCC ACC ATG CGA CAC AAA CGT TCT GCA AAA C- 3' (primer W; SEQ ID NO:5) and 5'-
- 15 GGC AGC CAA AGA GAC ATC TG-3' (primer X; SEQ ID NO:6). Oligomers 5'-CAG ATG TCT CTT TGG CTG CCA TGG AGC CAG TAG ATC CTA GAC-3'(primer Y; SEQ ID NO:7) and 5'-CTC GTA AGA TCT CTA TTC CTT CGG GCC TGT C-3'(primer Z; SEQ ID NO:8) were used to amplify the complete Tat open reading frame and to introduce sequence
- 20 complementarity with L2. The products from both PCR reactions were agarose gel isolated and then combined in a second PCR reaction in which only primers W and Z were added which generated the L2/Tat fusion through overlap of complementary sequences. These primers also appended Bgl II sites which were digested to facilitate insertion within
- 25 V1Jp to form V1Jp-L2cTat.

- Activity of the L2/Tat fusion was determined by transfection of V1Jp-L2cTat into P4R5 cells using a calcium phosphate transfection kit (available from Gibco BRL, Rockville, MD) performed as recommended by the manufacturer. P4R5 cells contain an integrated copy of the gene
- 30 encoding beta galactosidase under control of the HIV LTR. If the introduced Tat gene encodes functionally active Tat, expressed Tat protein will bind the LTR and result in expression of beta galactosidase. Lysates

were made of 48 hr post transfection cultures and beta galactosidase activity assessed using Galactostar® reagents (Tropix, Bedford, MS). The results are summarized in Figure 4 which shows that there were similar amounts of beta galactosidase produced in cells transfected with V1Jp-  
5 L2cTat as with the unfused positive control pD5-Tat plasmid (235 vs. 175). The no-Tat DNA control had a value of 0.462. Thus, full Tat activity appears to be retained within the L2/Tat fusion.



## WHAT IS CLAIMED IS:

1. A method of delivering a peptide or protein to a cell comprising:
  - a) fusing a first nucleic acid encoding the peptide or protein to a second nucleic acid encoding a modified human papillomavirus (HPV) L2 to create a fusion protein gene, wherein the L2 portion of the fusion protein gene is less than a full-length L2 gene, but encodes domains from the amino and the carboxyl termini;
  - b) expressing the fusion protein gene in a host cell to obtain fusion protein;
  - c) contacting the fusion protein with HPV L1 protein under conditions wherein the fusion protein and the L1 protein spontaneously combine to form a virus-like particle (VLP);
  - d) delivering the VLP to the cell.
2. A method according to Claim 1 wherein the HPV is selected from the group consisting of: HPV6a, HPV6b, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, and HPV56.
3. A method according to Claim 2 wherein the L2 gene encodes less than about 50% of wild type L2 protein.
4. A method according to Claim 2 wherein the peptide or protein to be delivered is selected from the group consisting of: HPV E1, E2, E3, E4, E5, E6, E7, HIV Tat and beta-lactamase.
5. A nucleic acid encoding a fusion peptide or protein comprising: a first segment comprising a nucleic acid encoding a portion of an HPV L2 protein which is less than a full-length sequence, and a second segment comprising a nucleic acid encoding a peptide or protein.

6. A nucleic acid according to Claim 5 wherein the first segment is from an HPV selected from the group consisting of:

HPV6a, HPV6b, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35,  
5 HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, and HPV56.

7. A nucleic acid according to Claim 6 wherein the second segment encodes a protein selected from the group consisting of:  
E1, E2, E3, E4, E5, E6, E7 HIV Tat and beta-lactamase.

8. A HPV virus-like particle (VLP) comprising HPV L1 protein and a fusion protein wherein the fusion protein comprises:

a first protein which is an HPV L2 protein which is less than  
15 about 50% of wild type; and  
a second protein or peptide.

9. A VLP according to Claim 8 wherein the HPV is selected from the group consisting of: HPV6a, HPV6b, HPV11, HPV16,  
20 HPV18, HPV31, HPV33, HPV35, HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, and HPV56.

10. A host cell comprising the VLP of Claim 8.

25 11. A vaccine comprising a virus-like particle according to Claim 10.

12. A nucleic acid encoding a VLP according to Claim 9.

1/4

Sma I

primer I >cttcccccgggcacaaaaacaa  
cttcccccgggcacaaaaacaa

atgc&gt;

ATGcgacacaaacgttctgcaaaacgcacaaaacgtgcatcggtaccaactttataaa  
acatgcaaacaggcaggtacatgtccacctgacattataacctaaggttgaaggcaaaact  
attgctgatcaaatattacaatatggaagtatgggtgtatttttgggtgggttaggaatt  
ggaacagggtcgggtacaggcggacgcactgggtatattccattgggaacaaggcctccc  
<cccagcccattgtccgcGgcgCTCGAGCTC> primer C

Not I Sac I

Xho I

acagctacagatacacttgctcctgtaagacccctttaacagtagatcctgtgggccct  
tctgatccttctatagtttcttttagtgaagaaactagttttattgatgctgggtgacca  
acatctgtaccttccatccccagatgtatcaggatttagtattactacttcaactgat  
accacacctgctatattagatattaataactgttactactgttactacacataataat  
cccactttcactgaccatctgtattgcagcctccaacacctgcagaaactggagggcat  
tttacactttcatcatccactattagtacacataaattatgaagaaattccctatggatata  
tttatgttagcacaaccctaacacagtaactagtagcacaccataccagggtctcgc  
ccagtgccacgcctaggatttatatagtcacacaacacaacagttaaagtgtgagccct  
gcttttgaaccactcccactaaacttattacatatgataatcctgcatatgaaggata  
gatgtggataatacattatattttctagtaattgataatagtattataatagctccagat  
cctgactttttggatatagtttgctttacataggccagcattaaaccttaggcgtactggc  
attaggtacagtagaattggtaataaacaacactacgtactcgtagtggaaaaatctata  
gggtgtaagggtacattattattatgatttgagtactattgatcctgcagaagaataagaa  
ttacaactataacaccttctacatatactaccacttcacatgcagcctcacctacttct  
attaataatggcttatatgatattttatgcagatgactttattacagatacttctacaacc

Not I Sac I

Xho I

primer A >GCGGCCGCGAGCTCGAGggttatattcctgcaaatataac>  
ccggtaccatctgtacccctctacatctttatcaggttatattcctgcaaatatacaaat  
ccttttgggtgcatacaaatattcctttagatcagggtcctgatataccattaatata  
actgaccaagctccttcatttaattcctatagttccagggtctccacaatatatacaattatt  
gctgatgcagggtgactttttattacatcctagtattacatgttacgaaaaacgacgtaaa  
cgtttaccatatttttttcagatgtcttttggtgccTAG

primer D <gtctacagagaaaccgacggatcTCTAGACCTCCC<

Bgl II

FIG. 1

09/762794

2/4

ATG CGA CAC AAA CGT TCT GCA AAA CGC ACA AAA CGT GCA TCG GCT ACC CAA CTT  
 M R H K R S A K R T K R A S A T Q L  
 1 10  
 TAT AAA ACA TGC AAA CAG GCA GGT ACA TGT CCA CCT GAC ATT ATA CCT AAG GTT  
 Y K T C K Q A G T C P P D I I P K V  
 20 30  
 GAA GGC AAA ACT ATT GCT GAT CAA ATA TTA CAA TAT GGA AGT ATG GGT GTA TTT  
 E G K T I A D Q I L Q Y G S M G V F  
 40 50  
 Not I. Sac I Xho I  
 TTT GGT GGG TTA GGA ATT GGA ACA GGG TCG GGT ACA GGC GGC CGC GAG CTC GAG  
 F G G L G I G T G S G T G G R ~~E~~ L ~~E~~  
 60 69 70 72  
 GGT TAT ATT CCT GCA AAT ACA ACA ATT CCT TTT GGT GGT GCA TAC AAT ATT CCT  
 G Y I P A N T T I P F G G A Y N I P  
 80 90  
 TTA GTA TCA GGT CCT GAT ATA CCC ATT AAT ATA ACT GAC CAA GCT CCT TCA TTA  
 L V S G P D I P I N I T D Q A P S L  
 100  
 ATT CCT ATA GTT CCA GGG TCT CCA CAA TAT ACA ATT ATT GCT GAT GCA GGT GAC  
 I P I V P G S P Q Y T I I A D A G D  
 110 120  
 TTT TAT TTA CAT CCT AGT TAT TAC ATG TTA CGA AAA CGA CGT AAA CGT TTA CCA  
 F Y L H P S Y Y M L R K R R K R L P  
 130 140  
 TAT TTT TTT TCA GAT GTC TCT TTG GCT GCC TAG  
 Y F F S D V S L A A  
 150 154

FIG. 2

3/4

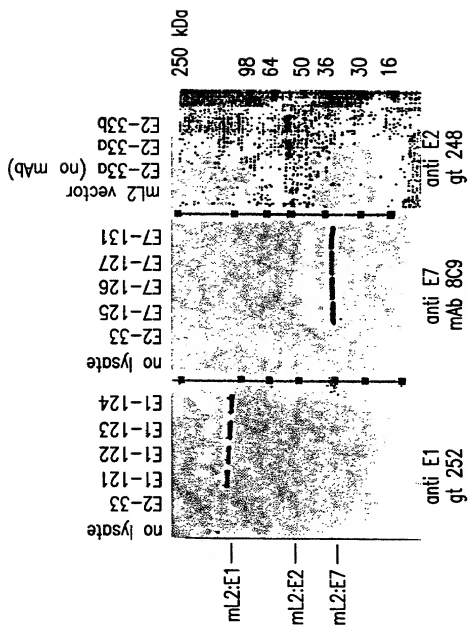
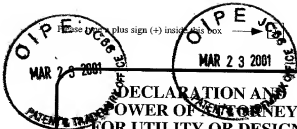


FIG.3

BETA-GALACTOSIDASE ACTIVITIES OF P4R5 CELL LYSATES TRANSFECTED WITH THE LISTED PLASMID CONSTRUCTS AND EVALUATED USING THE GALACTOSTAR<sup>®</sup> ASSAY

SAMPLE	BETA-GALACTOSIDASE RELATIVE LIGHT UNITS
V1jp-L2cTot	229.44
pD5 Tot (POSITIVE CONTROL)	196.48
MOCK TRANSFECTION (NEGATIVE CONTROL)	0.40

FIG.4

**DECLARATION AND  
POWER OF ATTORNEY  
FOR UTILITY OR DESIGN  
PATENT APPLICATION  
(37 CFR 1.63)**

☐ Declaration Submitted with Initial Filing **OR** ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	20276P
First Named Inventor	LOWE, ET AL.
<b>COMPLETE IF KNOWN</b>	
Application Number	09/762,794
Filing Date	February 9, 2001
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**PROTEIN DELIVERY SYSTEM USING HUMAN PAPILLOMAVIRUS VIRUS-LIKE PARTICLES**

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 08/14/1999 as United States Application Number or PCT International

Application Number PCT/US99/17931 and was amended on (MM/DD/YYYY) ( ) (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Attorney Docket Number	Priority Claimed?	
				YES	NO
				<input type="checkbox"/>	<input type="checkbox"/>
				<input type="checkbox"/>	<input type="checkbox"/>
				<input type="checkbox"/>	<input type="checkbox"/>
				<input type="checkbox"/>	<input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	Attorney Docket Number
60/096,638	08/14/1998	20276PV

**DECLARATION AND POWER OF ATTORNEY for Utility or Design Patent Application**

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information known to me to be material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Application Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint, respectively and individually, as my attorneys or agents with full power of substitution and revocation, the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

☐ Customer Number

OR

☒ Registered practitioner(s) name/registration number listed below

Place Customer Number  
Bar Code Label here

Name	Registration Number	Name	Registration Number
JOANNE M. GIESSER	32,838	JACK L. TRIBBLE	32,633

Direct all correspondence to: ☒ Customer Number or Bar Code Label

000210

<b>Name</b>	JOANNE M. GIESSER					
<b>Address</b>	Merck & Co., Inc. - Patent Department					
<b>Address</b>	P.O. Box 2000, RY60-30					
<b>City</b>	Rahway		<b>State</b>	NJ	<b>ZIP</b>	07065-0907
<b>Country</b>	USA	<b>Telephone</b>	(732)594-3046		<b>Fax</b>	(732)594-4720

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

**Name of Sole or First Inventor:**

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

ROBERTS

LOWE

**Inventor's Signature**

X Robert S. Lowe

**Date**

X 07 Feb 2001

**Residence: City**

NORTH WALES

**State** PA

**Country** US

**Citizenship** US

**Post Office Address**

Merck & Co., Inc., P.O. Box 2000

**City**

Rahway

**State** NJ

**ZIP** 07065-0907

☒ Additional inventors are being named on the \_\_\_\_\_ supplemental Additional Inventors(s) sheet(s) PTO/SB/02A attached hereto.



## DECLARATION AND POWER OF ATTORNEY

ADDITIONAL INVENTOR(S)  
Supplemental Sheet

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
KATHRIN U.				JANSEN			
Inventor's Signature		X <i>[Signature]</i>		Date		X 07 February 2001	
Residence: City		DOYLESTOWN		State PA		Country US	
Post Office Address		Merck & Co., Inc., P.O. Box 2000					
City		Rahway		State NJ		ZIP 07065-0907	
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
JOSEPH G.				JOYCE			
Inventor's Signature		X <i>Joseph G. Joyce</i>		Date		X 07 Feb 2001	
Residence: City		LANSDALE		State PA		Country US	
Post Office Address		Merck & Co., Inc., P.O. Box 2000					
City		Rahway		State NJ		ZIP 07065-0907	
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
WILLIAM L.				MCCLEMENTS			
Inventor's Signature		X <i>William L. McClements</i>		Date		X 07 Feb 2001	
Residence: City		DOYLESTOWN		State PA		Country US	
Post Office Address		Merck & Co., Inc., P.O. Box 2000					
City		Rahway		State NJ		ZIP 07065-0907	
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
JAMES C. III				COOK			
Inventor's Signature		X <i>James C. Cook</i>		Date		X Feb 7 2001	
Residence: City		LANSDALE		State PA		Country US	
Post Office Address		Merck & Co., Inc., P.O. Box 2000					
City		Rahway		State NJ		ZIP 07065-0907	

## DECLARATION AND POWER OF ATTORNEY

ADDITIONAL INVENTOR(S)  
Supplemental Sheet

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
JESSICA CHING-YEE				LING			
Inventor's Signature	<i>Jessica Ching-Yee</i>			Date	X Feb 07, 2001		
Residence: City	BENSALEM PA	State	PA	Country	PA	Citizenship	US
Post Office Address	Merck & Co., Inc., P.O. Box 2000						
City	Rahway	State	NJ	ZIP	07065-0907		
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
MICHAEL P.				NEEPER			
Inventor's Signature	<i>Michael P. Neeper</i>			Date	X Feb 07, 2001		
Residence: City	COLLEGEVILLE	State	PA	Country	US	Citizenship	US
Post Office Address	Merck & Co., Inc., P.O. Box 2000						
City	Rahway	State	NJ	ZIP	07065-0907		
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Inventor's Signature				Date			
Residence: City		State		Country		Citizenship	
Post Office Address	Merck & Co., Inc., P.O. Box 2000						
City	Rahway	State	NJ	ZIP	07065-0907		
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Inventor's Signature				Date			
Residence: City		State		Country		Citizenship	
Post Office Address	Merck & Co., Inc., P.O. Box 2000						
City	Rahway	State	NJ	ZIP	07065-0907		
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Inventor's Signature				Date			
Residence: City		State		Country		Citizenship	
Post Office Address	Merck & Co., Inc., P.O. Box 2000						
City	Rahway	State	NJ	ZIP	07065-0907		

## SEQUENCE LISTING

<110> Merck & Co., Inc.

<120> PROTEIN DELIVERY SYSTEM USING HUMAN  
PAPILLOMAVIRUS VIRUS-LIKE PARTICLES

<130> 20276 PCT

<150> 60/096,638

<151> 1998-08-14

<160> 8

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 1

cttccccccg ggcacaaaac aaaatgc

27

<210> 2

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 2

ctcgagctcg cggccgcctg tacccgaccc

30

<210> 3

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 3

gcggccgcga gctcgagggt tatattcctg caaatacaa

39

<210> 4

<211> 35

<212> DNA

<213> Artificial Sequence

<220>  
 <223> PCR primer  
  
 <400> 4  
 ccctccagat ctctaggcag ccaaagagac atctg 35  
  
 <210> 5  
 <211> 46  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> PCR primer  
  
 <400> 5  
 tccccggga gatctgccac catgcgacac aaacgttctg caaaac 46  
  
 <210> 6  
 <211> 20  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> PCR primer  
  
 <400> 6  
 ggcagccaaa gagacatctg 20  
  
 <210> 7  
 <211> 42  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> PCR primer  
  
 <400> 7  
 cagatgtctc ttggctgcc atggagccag tagatcctag ac 42  
  
 <210> 8  
 <211> 31  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> PCR primer  
  
 <400> 8  
 ctcgtaagat ctctattcct tcgggectgt c 31